Endogenous Testosterone Controls Humoral Immunity in the Lizard, *Chalcides ocellatus*

ABDEL HAKIM SAAD, MOHAMED HESHAM MANSOUR,

MANSOUR EL YAZJI and NASRI BADIR

Zoology Department, Faculty of Science, Cairo University, Cairo 12613, Egypt

ABSTRACT—Study of the correlation between endogenous blood levels of testosterone (TS) and the humoral immune response of male lizards, *Chalcides ocellatus* led to three major findings: 1) Long-term testosterone propionate (TP) treatment in "summer" induced an elevation of serum TS and complete abrogation of primary anti-rat erythrocytes (RRBC) antibody response similar to normal intact lizards during "spring"; 2) Bilateral orchidectomy of male lizards during "spring" showed increased response to RRBC and suggested that orchidectomy potentiated immunity and morc particularly definite stimulation of humoral immunity; 3) Long-term TP treatment of orchidectomized lizards failed to suppress immune response to RRBC. Indeed, orchidectomized lizards given TP had response to RRBC similar to male intact controls. The results are discussed from the perspective of the role played by TS in mediating the seasonal rhythms that affect reptilian immunity.

INTRODUCTION

Many decades ago scientists and clinicans alike observed that there were striking differences between the immune responsiveness of males and that of females [1]. In general, female had superior humoral and cell-mediated immunity [2, 3]. It is very likely that this difference in the immune response between the sexes is mediated by the action of sex steroids hormones on the immunoreactive cell populations [4]. Since the major source of these sex steroids is the gonads, it follows that removal of these organs by gonadectomy might be expected to alter the immune response. Although gonadectomy has been shown to alter the cell-mediated immune response, there is a considerable controversy concerning the effects of gonadectomy on antibody production [5-9]. In contrast to the wealth of information obtained from study of gonadectomy and immunocompetence of mammalian vertebrates, data available about fish [10] and amphibians [11] are inadequate and as yet limited. At the reptilian level, no

Accepted June 18, 1992 Received February 14, 1992. attempts were made to study the influence of gonadectomy on different immunological parameters.

The present study is mainly based on previous observations from our laboratory showing that the immune system of the lizard, *Chalcides ocellatus* was moderately active during April throughout June, reaching its maximum development during July/August [12, 13]. This slowly vernal building of immune response was ascribed to be due to high levels of endogenous testosterone (TS) [12]. Therefore, the present study was initiated to examine the effect of adult orchidectomy and testosterone treatment on the immune response of *C. ocellatus*. This study was conducted in view of contributing further information to the endogenous hormonal factors that mediate seasonal changes in immunity of reptiles.

MATERIALS AND METHODS

Lizards

Sexually mature male lizards, *Chalcides ocellatus* (Scincidae, non-hibernator, viviparous), 30–40 g in weight were collected from gradens, fields and arid regions in the vicinity of Cairo. Lizards were maintained as described previously [12] and given wood lice and tap water *ad libitum*. The study was performed from March through September (temperature ranges of 25–38°C).

Preparation of cell suspensions

Lymphoid cells were separated from lizard spleen exactly as described in detail earlier [13]. After three washes in cold RPMI 1640 medium (Sigma Chemicals Co., St. Louis, MO., USA), lymphocytes were counted using the trypan blue dye exclusion test.

Blood testosterone (TS) determination

Serum testosterone was measured following essentially the method described earlier [12] using a commercial radio-immunoassay kit (Coat-A-Count, Diagnostic Products Corporation, CA, ¹²⁵I-labelled TS was supplied as the USA). radioactive tracer. Tetosterone showed less than 1.5% cross reactivity with corticosterone, a major serum corticosteroids in C. ocellatus [13]. The mean intra- and interassay coefficient of variation of assays fell within the range of 4.0-7.0% and 8.0-15.0%, respectively. In these assays, the minimum detectable dose on the standard curve was 10 pg. Recovery of labelled and unlabelled steroids standard was 90-93%. Values shown in the "Results" are corrected for recovery.

Primary immunization with RRBC

Animals were allowed to acclimate to ambient environmental conditions for few days before immunization. The lizards received intraperitoneal (i.p.) immunizing injections of 0.5 ml of 10% rat erythrocytes (RRBC) suspension in phosphatebuffered saline (PBS), pH=7.2. Unimmunized (control) lizards were injected i.p. with 0.5 ml PBS, pH=7.2 and included in every such experiments. After sacrifice by decapitation, blood was collected and allowed to clot at room temperature. Serum was then heat-inactivated at 56°C for 30 min. and stored for use in the haemagglutination test.

Rosette-forming cell (RFC) assay

Groups of animals were sacrificed at a specified

intervals after immunization. A single cell suspension from the spleen was prepared as described and the viability was determined by trypan blue exclusion method. The rosette-forming cell (RFC) assay was followed to assess the number of antigen-binding cells as described earlier [14]. On the basis of actual number of cells for each sample, the RFC per 10^6 spleen cells was computed for each spleen.

Plaque-forming cell (PFC) assay

The technique of Cunningham and Szenberg [15] as modified for lizards by Kanakambika and Muthukkaruppan [16] was followed to assess the number of haemolytic antibody-producing cells. Plaques were counted under low power with the aid of a dark field phase contrast microscope. The number of PFC/ 10^6 viable spleen cells was calculated.

Determination of antibody (Ab)

Haemagglutination (HA) titrations of the sera were performed in microtiter plates using two-fold serial dilution as described in detail previously [12]. Titers were expressed as the \log_2 of the highest dilution showing microscopic agglutination.

Bilateral orchidectomy

Adult male lizards were allowed to acclimate to laboratory conditions for at least one week before operation. Surgical operations were performed under ether anaethesia. Operation sites were washed with distilled water followed by 70% cthyl alcohol. Skin was firstly cut by a sharp cissors, while the abdominal muscles cut by means of a small scalpel. The left then the right testes were separated from their blood supply by means of a cautery then cut off their respective vasa deferentia. Sulpha and neomycin was sprayed on the wound then sewed by means of a small curved surgical needle. Operated animals were placed in a clean glass terrarium for one day to avoid sepsis. In corresponding sham-operated controls, the testes were delivered to the wound surface, returned to the abdominal cavity and the wound closed. No death occurred in either operated or sham-operated groups during the course of the

1038

experiment. Both were kept at ambient temperature under normal conditions in the laboratory and given live wood lice and water *ad libitum*.

Testosterone propinate (TP) treatment

Intact, sham-operated or orchidectomized lizards were allowed to acclimate to ambient environemtnal conditions a few days before treatment. All lizards received at 5 day-intervals a total of 5 i.p. injections of 50 μ g testosterone propionate (Sigma)/g body weight/injection. Another group of lizards was injected at the same intervals with 0.3 ml of PBS, pH =7.2 and used as control. No death occurred in either TP-treated or control groups during the course of the experiment. Both were kept at ambient temperature under normal conditions in the laboratory and given wood lice and water *ad libitum*.

Statistical analysis

Student's *t*-test was used to determine levels of significance between control and experimental groups. Differences were considered to be significant at P values < 0.05.

RESULTS

Effect of long-term TP treatment on intact lizards: Circulating TS levels:

As illustrated in Figure 1, TS levels of intact males injected with PBS exhibited minor changes within the normal range of about 9.5–26.3 ng/ml. However, TS levels after multiple injections of TP were markedly elevated to the range of 18–140 ng/ml, and stabilized at this level until the end of the experiment. Ten days after cessation of TP, a decline to basal values proceeded sharply.

Primary anti-RRBC response:

In mid-summer, male lizards were given five i.p. injections of TP (50 μ g/g body weight/injection; 5 days apart). Another group of male lizards received PBS and served as controls. Two days later, all TP-treated and PBS-injected lizards received an i.p. injection of RRBC suspension. RFC and PFC assays were performed on individual animals using spleen cell suspensions on different days after



FIG. 1. The levels of serum TS in intact lizards, C. ocellatus, administered with multiple injections of 50 μ g TP/g body weight/injection. During midsummer, lizards received 5 injections, 5 days-apart, of TP suspension. Each point depicts the level of TS of serum sample pooled each from two animals. Day O represents TS levels of intact lizards injected with PBS.

immunization. Titrations of serum Ab titers were carried out simultaneously.

As depicted in Figure 2A, TP-treated male lizards elicited a low number of RFC comparable to that enumerated in PBS-injected lizards. However, significant (P < 0.01) differences could be observed at the peak day (Fig. 2A). As shown in Fig. 2B, although the number of PFC higher in PBS-injected males than in TP-treated lizards, no significant differences could be observed at the peak day. Indeed, the shape of the curves was otherwise quite similar. As depicted in Figure 2C, in PBS-injected lizards, low levels of Ab titer was detected below background level on day 7. Therefore, the titer rose quickly exhibiting a peak at day 21 and remained elevated until day 28. However, in TP-treated lizards, Ab titer increased steadily reaching its peak value at day 21, then gradually declined.

Effect of bilateral orchidectomy on intact lizards: Circulating TS level:

Adult male lizards, C. ocellatus were collected



during early spring and some of these lizards were orchidectomized during mid-March. Another group was sham-operated and used as controls. Figure 3 illustrates the serum TS levels in male sham- operated and orchidectomized lizards during March through July. Sham- operated lizards exhibited high levels of TS being in the range of 110-140 ng/ml. However, bilateral orchidectomy in *C. ocellatus* lowered the level of TS to about 2– 18 ng/ml. These abrogated levels were sustained and lasted long, so that the mean values for all operated lizards, at any given point were significantly (P < 0.001) different from sham-operated controls.



FIG. 3. The levels of serum TS obtained from adult *C. ocellatus* after bilateral orchidectomy. Each point on the curve represents the level of TS obtained from an individual sample pooled from two animals. Day O represents TS levels of sham-operated lizards.

FIG. 2. Kinetics of RFC response (A), PFC response (B) and mean serum haemagglutinin titers (C) of normal lizards, *C. ocellatus*, administered with multiple injections of 50 µg TP/g body weight/injection. During mid-summer, lizards received 5 injections, 5 days-apart, of TP suspension. Two days later, lizards were received i.p. injection of 0.5 ml of 10% RRBC suspension. Each point represent the mean response of 4–6 separate animals and the vertical bars indicate standard error of the mean.
● Normal intact lizards, ●…● Normal intact lizards injected with TP. *=0.05<*P*<0.01 and NS = not significant.

1040

Primary anti-RRBC response:

Groups of adult lizards were sham-operated or orchidectomized during the second week of March. Two separated experiments were performed: the *first* after one month and the *second* after three months of operation. During each experiment, orchidectomized and sham-operated lizards were received a single i.p. injection of RRBC suspension on day 0. RFC and PFC assays were carried out using spleen cell suspension on different days after immunization. Titration of serum Ab levels were carried out simultaneously.

As depicted in Figure 4A, in sham-operated lizards, the number of RFC increased from background levels to a peak level on day 14 and then dropped sharply between day 21 and day 28. In contrast, both groups of orchidectomized lizards receiving RRBC suspension manifested a significant (P < 0.01) increase in RFC on day 7. The number of RFC remained elevated on day 14, then gradually declined. As shown in Figure 4B, administration of RRBC suspension elicited a peak response of PFC on day 14, then sharply declined. However, both groups of orchidectomized lizards showed a sparp (P > 0.01) increase in the number of PFC which was peaked on day 14 and then gradually decreased (Fig. 4B). When shamoperated lizards received RRBC suspension, low levels of Ab titer was detectable below background level on day 7 (Fig. 4C). Thereafter, the titer rose slowly exhibited a peak at day 21, then gradually declined at day 28. However, in both groups of orchidectomized lizards, a sharp increase in criculating Ab titer occurred in day 7. Thereafter, a gradual increase occurred in the Ab titer (Fig. 4C).

FIG. 4. Kinetics of RFC response (A), PFC response (B) and mean haemagglutinin titers (C) of *C. ocellatus* after bilateral orchidectomy. Animals were immumized i.p. on day 0 with 0.5 ml of 10% RRBC suspension. Each point represents the mean response of 4–6 separate animals and the vertical bars indicate standard error of the mean. *=0.05 < P <0.01 and NS=not significant. •••• sham-operated lizards; $\triangle - \triangle$ orchidectomized lizards, one month after operation; $\bigcirc - \bigcirc$ orchidectomized lizards, three months after operation. *=0.05 < P < 0.01and NS=not significant.



Effect of long-term TP-treatment on orchidectomized lizards: Circulating TS levels:

TP was administered to orchidectomized lizards to determine whether increasing the concentration of serum TS would result in the modulation of immune reactivity. Those lizards were orchidectomized during May. One month later, one group of orchidectomized lizards received five fractionated dose of TP ($50 \mu g/g$ body weight/injection; 5 days-apart). Control orchidectomized lizards received five i.p. injections of PBS.

As illustrated in Figure 5, TS levels of orchidectomized lizards injected with PBS exhi-



FIG. 5. The levels of serum TS in orchidectomized C. ocellatus administered with multiple injections of 50 μ g testosterone propionate (TP)/g body weight/ injection. One month after orchidectomy, lizards received 5 injections, 5 days-apart, of TP. Each point depicts the level of TS of serum sample pooled each from two animals. Day O represents TS levels of orchidectomized lizards injected with PBS.

FIG. 6. Kinetics of RFC response (A), PFC response
(B) and mean serum haemagglutinin titers (C) of orchidectomized *C. ocellatus* administered with multiple injections of 50 µg TP/g body weight/injection. One month after orchidectomy, lizards received 5 injections, 5 days-apart, of TP suspension. Two days after injection, lizards were received i.p. injection of 0.5 ml of 10% RRBC suspension.

mized lizards injected with TP. *=0.05 < P < 0.01and NS=not significant.



bited minor changes within the normal range of 1.1-5.7 ng/ml. In comparison, orchidectomized lizards receiving five injections of TP showed a sharp increase in serum TS levels to about 45–120 ng/ml which maintained until day 20.

Primary anti-RRBC response:

TP was administered to orchidectomized lizards to determine whether increasing TS levels would result in modulation of the immune response. Lizards were orchidectomized during May. One month later, one group of orchidectomized lizards received i.p. five fractionated dose of TP ($50 \mu g/g$ body weight/injection; 5 days-apart). Control orchidectomized lizards received i.p. five injections of PBS. Two days after the last injection, lizards received i.p. injection of RRBC suspension.

shown in Figure 6A, in TP-treated As orchidectomized lizards, the kinetics of RFC was similar to that demonstrated in orchidectomized control lizards. Moreover, no significant differences were essentially observed between the two animal groups at the peak day. As shown in Figure 6B, in both PBS-injected and TP-treated orchidectomized lizards, the shape of the curves was otherwise quite similar. Although both groups of lizards showed a high response, no significant differences were observed at the peak day. As depicted in Figure 6C, despite the fact that in TP-treated orchidectomized lizards, the peak of primary Ab response to RRBC was somewhat delayed and diminished in comparison with PBSinjected orchidectomized lizards, statistical differences in humoral response at some time point were essentially not significant.

DISCUSSION

In the present study experimental administration of exogenous TP in lizards produced changes in serum TS levels which closely mimicked those occurring as part of response of lizards to the seasonal environment [12]. As expected, longterm TP-treatment of intact summer lizards led to a complete and an irreversible abrogation of their proliferative response to RRBC. The results indicated that the cell(s) responsible for these functional alterations in lizards were particularly TPsensitive; the target cell(s), however, remained undefined. It is obvious that several doses of exogenous TP induced a high and more long standing elevation of TS resulting in a depressed immune reactivity, which simulates the natural situation lizards experienced annually in spring [12].

In view of the above, the authors hypothesized that lowering an animal's blood TS levels by "orchidectomy" did alter the immune response and that alterations might be opposite to those induced by the administration of exogenous TP. Thus, bilateral orchidectomy was performed to adult male C. ocellatus in early spring in order to suppress the high TS levels at that time of the year The immune reconstitution of "spring" [12]. lizards based on actually reproducing endocrine environment is theoretically possible but experimentally difficult maneuver. Yet, the outcome has been fruitful. Although bilateral orchidectomy strongly reduced serum TS levels in C. ocellatus, it did not result in its complete disappearance. However, these concentrations were similar to the low values previously recorded throughout summer months [12]. Moreover, orchidectomized lizards showed increased response to RRBC and the rate of lymphoid tissue hyperplasia correlated with this increased reactivity (data not shown). therefore. suggested These data. that orchidectomy potentiated immunity in general and more particularly humoral one. Whether this effect is due to the differentiation of lymphoid precursor cells to immunocompetent cells and/or the proliferation of pre-existing competent cells, which might be present in low level in the other lymphoid organs is difficult to decide.

Although TS deprivation showed to cause immunological potentiation, the central point remaining is whether the effects are due directly to withdrawal of TS or they result from interference with the hormonal milieu. Therefore, TP was administered to orchidectomized lizards to determine whether increasing the concentration of blood TS would result in modulation of the immune reactivity. One month after orchidectomy, orchidectomized lizards received five fractionated doses of TP. These orchidectomized lizards exhibited a spring-like sustained rise of serum TS levels until the end of the experiment. However, physiologic doses of TP used in the current study failed to suppress immune responses to RRBC in orchidectomized lizards. In fact, orchidectomized lizards given TP had response to RRBC which were similar to male intact controls. These findings suggested that the presence of TS caused immune function to be "normal" in orchidectomized lizards.

To our knowledge, no such study has been available in reptiles. However, the scientific literature dealing with sex hormones effect on vertebrate immune responses appeared to be confused and scant. While in marine teleost, Sebasticus marmaratus, the humoral reactivity of mature females to RRBC was lower than males or immature females in the spawning season, yet exogenous administration of sex hormones or orchidectomy did not display influence on Ab production during the mating season [10]. The thymus and spleen were morphometrically analyzed in orchidectomized frogs, Rana perezi [11]. However, the authors failed to interpret their data, since such important physiological processes were undobtly the direct effect of TS deprivation [11]. In addition, recent studies indicated that neonatal gonadectomy in chicken exerted two effects on the humoral immune response: suppressive in young birds [17] and stimulatory in older chicken [18]. Moreover, orchidectomy resulted in immunopotentiation in many mammalian systems. For example, orchidectomy increased protection against viral or fungal [5-7] and bacterial infections [8] and parasitic infections [9]. Moreover, orchidectomized animals reject allografts rapidly [19] and accelerated graft-versus-host reactions [20]. Furthermore, gonadectomy augmented Tlymphoblasts transformation in culture [21] and potentiated humoral immune response to several heteroantigens [22, 23]. However, in some experimental situations, orchidectomy had no effect on the immune system [8].

In conclusion, all these results confirm the importance of sex hormones as causative agents of the seasonal variations of reptilian immune system and emphasized the lymphocyte destruction as the main effect of sex hormones on the immune system of lower vertebrates. Mechanisms of action of sex hormones, target cells for sex hormones in the lymphoid organs and the way of interaction between sex hormones and the immune axis in lower vertebrates deserve further research.

REFERENCES

- Ansar, Ahmed, S., Penhale, W. J. and Talal, N. (1985) Sex hormones, immune response and autoimmune diseases. Mechanisms of sex hormones action. Am. J. Pathol., 121: 531–551.
- 2 Grossman, C. J. (1984) Regulation of the immune system by sex steroids. Endocrine Rev., 5: 438-455.
- 3 Stimson, W. H. (1987) Sex steroids, steroid receptors and immunity. In "Hormones and Immunity". Ed. by I. BERCZI and K. KOVACS, MTP Press, Lancaster.
- 4 Berczi, 1. (1989) Immunoregulation by neuroendocrine factors. Dev. Comp. Immunol., 13: 329–341.
- 5 Berkoulch, S. and Ressel, M. (1967) Effect of sex on susceptibility of adult mice to coxackie B virus infections. Arch. Gesmate Virus Forsch, **22**: 246– 251.
- 6 Rifkind, D. (1972) Influence of gonadectomy on *Candida albicans* urinary tract infection in CFW mice. Infect. Immunol., 5: 363–369.
- 7 Streng, C. B. and Nathan, P. (1973) The immunc response in steroid deficient mice. 1mmunology, 24: 559–563.
- 8 Cohen, D. A. (1979) Sensitivity to androgen. A possible factor in sex differences in the immune response. Clin. Exp. Immunol., **38**: 218–225.
- 9 Kittas, C. and Henry, L. (1979) Effect of scx hormones on the immune system of guinea pigs and on the development of toxoplasmic lesions in nonlymphoid organs. Clin. Exp. Immunol., 36: 16–20.
- 10 Nakanishi, T. (1986) Scasonal changes in the humoral immune response and the lymphoid tissues of the marine teleost, *Sebastiscus marmoratus*. Vet. Immunol. Immunopathol., **12**: 336–342.
- 11 Zapata, A., Garrido, E. Gomariz, R. P. and Lcceta, J. (1983) Relationship between neuroendocrine and immune systems in amphibians and reptiles. Dev. Comp. Immunol., 7: 771–774.
- 12 Saad, A.-H., Abdel Khalik, N. and El, Ridi, R. (1990) Blood testosterone level: a seasondependent factor regulating immune reactivity in lizards. Immunobiol., 180: 184–190.
- 13 Saad, A.-H. and EL, Ridi, R. (1989) Endogenous corticosteroids mediate seasonal cycle changes in immunity of lizard. Immunobiol., 177: 390–403.
- 14 Saad, A.-H. and Shoukrey, N. (1988) Scxual dimorphism on the immune responses of the snake, *Psammophis sibilans*. Immunobiol., 177: 404–419.

- 15 Cunningham, A. and Szenberg, A. (1968) A further improvements in the plaque-technique for detecting single antibody forming cells. Immunology, 14: 599 -600.
- 16 Kanakambika, P. and Muthukkaruppan, V. R. (1972) The immune response to sheep erythroeytes in the lizard, *Calotes versicolor*. J. Immunol. 109: 415–419.
- 17 Vujie, D., Milieevic, Z., Mieie, M., Isakovie, K. and Jankovie, B. D. (1983) Atrophy of the thymus and bursa of Fabricius caused by neonatal gonadectomy. Period biol., 85: 107–109.
- 18 Milicevic, Z., Micic, M. and Isakovic, K. (1986) The role of gonads in immunomodulation and aging of the immune system. Period. biol., 88: 13–14.
- 19 Graff, R. J., Lappe, M. A. and Snell, G. D. (1969)

The influence of the gonads and the adrenal glands on the immune response to skin grafts. Transplant., 7: 105–111.

- 20 Castro, J. E. (1974) The hormonal mechanism of immuno-modulation in mice after orchidectomy. J. Endocrinol., 62: 311–318.
- 21 Grossman, G. J., Sholiton, L. G. and Roselle, G. (1982) Estradiol regulation of thymic lymphocyte function in the rat: mediation by serum thymic factors. J. Steroid Biochem., 16: 683–688.
- 22 Eidinger, D. and Garret, T. J. (1972) Studies of the regulatory effects of the sex hormones on antibody formation and stem cell differentiation. J. Exp. Med., 136: 1098–1116.
- 23 Castro, J. E. (1975) Immunological effects of orchidectomy. Br. J. Urol., 47: 89–96.